

## Primary research

**Simultaneous identification of *GSTP1* Ile105→Val105 and Ala114→Val114 substitutions using an amplification refractory mutation system polymerase chain reaction assay: studies in patients with asthma**

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(Print ISSN 1465-9921; Online ISSN 1465-993X)**Abstract****Background:** The glutathione S-transferase (GST) enzyme *GSTP1* utilizes byproducts of oxidative stress. We previously showed that alleles of *GSTP1* that encode the Ile105→Val105 substitution are associated with the asthma phenotypes of atopy and bronchial hyperresponsiveness (BHR). However, a further polymorphic site (Ala114→Val114) has been identified that results in the following alleles: *GSTP1*\*A (wild-type Ile105→Ala114), *GSTP1*\*B (Val105→Ala114), *GSTP1*\*C (Val105→Val114) and *GSTP1*\*D (Ile105→Val114).**Methods:** Because full identification of *GSTP1* alleles may identify stronger links with asthma phenotypes, we describe an amplification refractory mutation system (ARMS) assay that allows identification of all genotypes. We explored whether the *GSTP1* substitutions influence susceptibility to asthma, atopy and BHR.**Results:** Among 191 atopic nonasthmatic, atopic asthmatic and nonatopic nonasthmatic individuals, none had the BD, CD, or DD genotypes. *GSTP1* BC was significantly associated with reduced risk for atopy ( $P=0.031$ ). Compared with AA, trend test analysis identified a significant decrease in the frequency of *GSTP1* BC with increasing severity of BHR ( $P=0.031$ ). Similarly, the frequency of *GSTP1* AA increased with increasing BHR.**Conclusion:** These data suggest that *GSTP1*\*B and possibly *GSTP1*\*C are protective against asthma and related phenotypes.**Keywords:** amplification refractory mutation system, asthma, bronchial hyperresponsiveness, *GSTP1***Introduction**

Polymorphisms in members of the GST supergene family have been associated with individual susceptibility to lung diseases [1]. In the context of asthma *GSTP1* – the predominant GST expressed in human lung [2] – is a candidate because this enzyme has a role in cellular protection

against oxidative stress [3]. Thus, *GSTP1* catalyzes the detoxification of byproducts of lipid and DNA oxidation [1]. Asthma is characterized by airway inflammation [4]. Indeed, BHR reflects the presence of inflammation, and is exhibited by virtually all asthmatic patients. Atopic individuals (as defined by serum IgE levels and skin prick tests)

ARMS = amplification refractory mutation system; BHR = bronchial hyperresponsiveness; CI = confidence interval; FEV<sub>1</sub> = forced expiratory volume in 1 s; GST = glutathione S-transferase; OR = odds ratio; PC<sub>20</sub> = provoking concentration of an inhaled substance that causes a 20% reduction in FEV<sub>1</sub>; PCR = polymerase chain reaction.

Table 1

## Clinical parameters of recruited subjects

Parameters	FEV <sub>1</sub> >80% predicted*			
	BHR negative (PC <sub>20</sub> >16 mg/ml; group 1)	BHR positive (PC <sub>20</sub> 8–16 mg/ml; group 2)	BHR positive (PC <sub>20</sub> 0.03–8 mg/ml; group 3)	FEV <sub>1</sub> ≤80% predicted* (group 4) <sup>†</sup>
<i>n</i> (%)	58 (33.9)	15 (8.8)	51 (29.8)	47 (27.5)
Mean age ± SD (years)	37.0 ± 11.9	29.3 ± 6.8	33.0 ± 11.1	41.4 ± 11.0
Males ( <i>n</i> [%])	18 (31.0)	4 (26.7)	13 (25.5)	16 (34.0)
Females ( <i>n</i> [%])	40 (69.0)	11 (73.3)	38 (74.5)	31 (66.0)
Skin test negative ( <i>n</i> [%])	32 (55.2)	7 (46.7)	12 (23.5)	7 (14.9)
Skin test positive ( <i>n</i> [%])	26 (44.8)	8 (53.3)	39 (79.5)	40 (85.1)
IgE ≤100 IU/ml* ( <i>n</i> [%])	38 (71.7)	10 (83.3)	21 (46.7)	16 (40.0)
IgE >100 IU/ml* ( <i>n</i> [%])	15 (28.3)	2 (16.9)	24 (53.3)	24 (60.0)

\*In some patients we were unable to obtain a complete data set. Some individuals were unwilling to undertake a metacholine challenge test, whereas others did not wish to donate a blood sample (in this case DNA was isolated from a mouthwash sample). <sup>†</sup>Mean FEV<sub>1</sub> = 67% predicted (min = 45%, max = 78%).

are very likely to have increased airway responsiveness [4]. Thus, studies designed to identify susceptibility genes for asthma must consider the possible interrelationship of BHR and atopy in the expression of the asthma phenotype.

We previously showed that the Ile105→Val105 substitution in *GSTP1* is strongly associated with severity of BHR [5]. A further polymorphism is present at amino acid 114 (Ala114→Val114), however, indicating that unequivocal identification of *GSTP1* alleles requires consideration of both substitutions. These polymorphisms give rise to wild-type *GSTP1*\*A (Ile105→Ala114), *GSTP1*\*B (Val105→Ala114), *GSTP1*\*C (Val105→Val114) and *GSTP1*\*D (Ile105→Val114) [6–9]. Although the Ile105 variant has a higher catalytic efficiency for 1-chloro-2,4-dinitrobenzene than does the Val105 variant [6], the Val105 variant appears to confer higher catalytic efficiency for polycyclic aromatic hydrocarbon diol epoxides [7–11]. The effect of the Ala114→Val114 substitution is unclear, although it may enhance the effect of the Ile105→Val105 substitution [11].

Because the substitution at amino acid 114 may modify the association of *GSTP1* Ile105→Val105 with asthma phenotypes, we developed an ARMS assay in order to identify unambiguously those genotypes that result from the A, B, C and D alleles. This approach is necessary, because presently described assays do not differentiate AC and BD genotypes. We also determined the frequencies of these genotypes in atopic nonasthmatic, atopic asthmatic and nonatopic nonasthmatic healthy persons.

## Materials and methods

### Patients

Unrelated Northern European nonatopic nonasthmatic, atopic nonasthmatic and atopic asthmatic persons (*n* = 191) were recruited in North Staffordshire, UK [5]. They were stratified by degree of airway reactivity/obstruction as follows (Table 1): group 1, BHR negative (normal), with forced expiratory volume in 1 s (FEV<sub>1</sub>) greater than 80% predicted and PC<sub>20</sub> greater than 16 mg/ml metacholine; group 2, borderline BHR (mild) with FEV<sub>1</sub> greater than 80% predicted and PC<sub>20</sub> positive with 8–16 mg/ml metacholine; group 3, BHR positive (moderate) with FEV<sub>1</sub> greater than 80% predicted and provoking concentration of an inhaled substance that causes a 20% reduction in FEV<sub>1</sub> (PC<sub>20</sub>) positive with 0.03–8 mg/ml metacholine; and group 4, severe airway dysfunction, with FEV<sub>1</sub> of 80% or less than predicted, which was not challenged with metacholine for ethical reasons. A positive skin reaction (mean wheal diameter of at least 3 mm more than with saline control) in response to at least one of a panel of seven common aeroallergens (house dust mite, house dust, grass mix, tree pollen, cat fur, dog fur, feathers) and serum IgE levels greater than 100 IU/ml were used to define atopic status, together with personal history. The local Ethics Committee approved the study, and all participants provided written informed consent.

### Determination of *GSTP1* genotypes

Genotyping was performed using leucocyte DNA. The Ile105→Val105 substitution was identified using primers to exon 5 as previously described [7,12]. The Ala114→Val114 substitution was identified as described

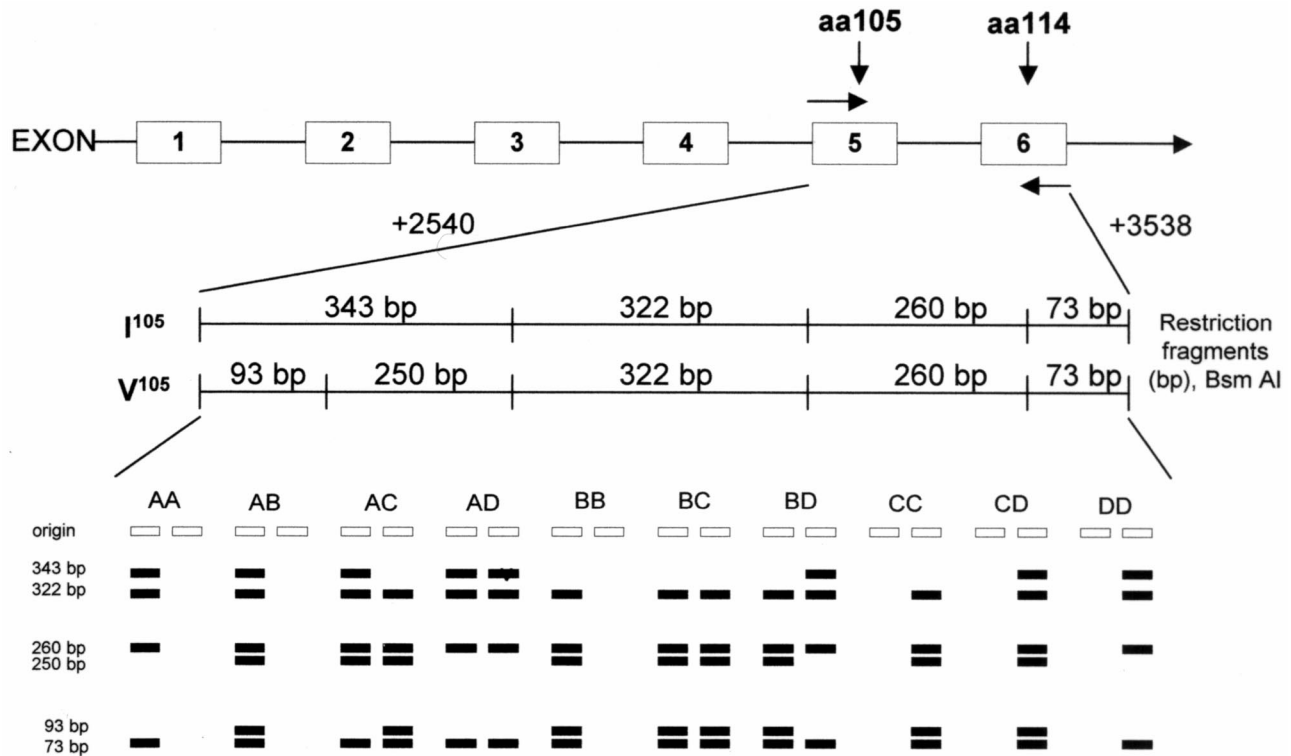
**Figure 1**

Illustration of the sites of primers and *Bsm* AI restriction endonuclease maps of the variant *GSTP1*, together with the banding patterns obtained from the ARMS assay for each genotype. Products gave fragments of 343, 322, 260 and 73 base pairs with Ile105/Ile105; 93, 250, 322, 260 and 73 base pairs with the Val105/Val105; and all six fragments in heterozygotes (Ile105/Val105). Base pair numbering according to Morrow *et al* [21].

by Board *et al* [13], with an annealing temperature of 65°C. However, *GSTP1* AC and BD gave heterozygous patterns using both of the above assays. An ARMS assay was therefore developed to differentiate between these genotypes. This included a forward primer upstream of the codon 105 substitution (5'-ACCCAGGGCTC-TATGGGAA-3') and two reverse primers (primer A [Ala114 specific], 5'-TCACATAGTCATCCTTGCCGG-3'; and primer B [Val114 specific], 5'-TCACATAGTCATC-CTTGCCGA-3').

For each DNA sample two polymerase chain reactions (PCRs) were performed, amplifying a 998 base pair fragment. PCRs were carried out in 50 µl containing forward primer, reverse primer A or B (2 × 0.25 µmol/l), Taq polymerase (1 U), dNTP (4 × 200 µmol/l), 1 × polymerase buffer (10 mmol/l Tris-HCl pH 9.0, 50 mmol/l KCl, 0.1% [vol/vol] Triton X-100, 1.5 mmol/l MgCl<sub>2</sub>), and target DNA (approximately 0.5 µg). Conditions were as follows: 94°C for 4 min, 30 cycles of denaturation (94°C, 1 min), primer annealing (62°C, 1 min) and elongation (72°C, 2 min).

The initial ARMS PCR was used to determine the Ala114→Val114 genotype. Thus, in persons with

Ala114/Ala114 amplification occurred only with primer A, and only with primer B in persons with Val114/Val114; for Ala114/Val114 heterozygotes, amplification occurred with both primers. ARMS PCR products were then digested with *Bsm* AI to determine the *cis/trans* configuration (which variant at position 105 is paired with which allele at position 114) of the Ile105→Val105 encoding allele and resolved in 2% (vol/weight) agarose gels. Products gave fragments of 343, 322, 260 and 73 base pairs with Ile105/Ile105; 93, 250, 322, 260 and 73 base pairs with Val105/Val105; and all six fragments in heterozygotes (Ile105/Val105; Fig. 1).

Every run (including both restriction fragment length polymorphism and ARMS assays) included DNA samples of known genotype as positive controls and one negative control (no DNA). Samples with AA (Ala114/Ala114) and CC (Val114/Val114) were used to optimize the procedure, and as controls for the ARMS PCR. As an assessment of quality control, approximately 15% of DNA samples were reassayed at least once in order to confirm the assigned genotype. All results from the reassayed samples were consistent with the original genotype assignment.

Table 2

The association of *GSTP1* genotype with atopic status and degree of airway reactivity/obstruction

Genotype	Total group	Skin test negative	Skin test positive	IgE ≤ 100 IU/ml	IgE > 100 IU/ml	Group 1	Group 2	Group 3	Group 4
AA	79 (42.4)	29 (40.9)	50 (41.7)	42 (43.3)	27 (38.6)	17 (29.3)	4 (26.7)	21 (41.2)	23 (48.9)
AB	66 (34.6)	21 (29.6)	45 (37.5)	30 (30.9)	30 (42.9)	24 (41.4)	6 (40.0)	21 (41.2)	14 (29.8)
AC	26 (13.6)	10 (14.1)	16 (13.3)	11 (11.3)	11 (15.7)	7 (12.1)	3 (20.0)	6 (11.8)	7 (14.9)
AD	2 (1.1)	0 (0)	2 (1.7)	1 (1.0)	1 (1.4)	1 (1.7)	0 (0)	0 (0)	1 (2.1)
BB	7 (3.7)	4 (5.6)	3 (2.5)	5 (5.2)	0 (0)	3 (5.2)	1 (6.7)	1 (2.0)	1 (2.1)
BC	9 (4.7)	7 (9.9)	2 (1.7)*	7 (7.2)	0 (0)	5 (8.6)	1 (6.7)	1 (2.0)	1 (2.1)
BD	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
CC	2 (1.1)	0 (0)	2 (1.7)	1 (1.0)	1 (1.4)	1 (1.7)	0 (0)	1 (2.0)	0 (0)
CD	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
DD	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Alleles									
<i>GSTP1</i> *A	0.660	0.627	0.679	0.649	0.686	0.569	0.567	0.676	0.731
<i>GSTP1</i> *B	0.233	0.254	0.221	0.242	0.214	0.302	0.300	0.235	0.172
<i>GSTP1</i> *C	0.102	0.120	0.092	0.103	0.093	0.121	0.133	0.088	0.086
<i>GSTP1</i> *D	0.005	0	0.008	0.005	0.007	0.009	0	0	0.011

Atopic status was defined as positive skin test and IgE 100 IU/ml or less. Groups were defined as follows: group 1, percentage predicted >80%/PC<sub>20</sub> >16 mg/ml; group 2, percentage predicted >80%/PC<sub>20</sub> 8–16 mg/ml; group 3, percentage predicted >80%/PC<sub>20</sub> 0–8 mg/ml; and group 4, percentage predicted ≤80%. Values are expressed as number (%) for the genotype frequencies, or as proportions for the allele frequencies. \*Logistic regression analysis for skin test: uncorrected *GSTP1* BC versus *GSTP1* AA, OR 0.17, 95% CI 0.03–0.85 ( $P = 0.031$ ); and corrected for age and sex, OR 0.18, 95% CI 0.04–0.96 ( $P = 0.045$ ). Trend test analysis across groups 1–4: uncorrected *GSTP1* BC versus *GSTP1* AA,  $P = 0.031$ ; and corrected for age and sex,  $P = 0.02$ .

## Statistical analysis

$\chi^2$  tests were used to assess homogeneity between groups (eg skin test positive versus skin test negative). As some allele frequencies were small, the StatXact-Turbo (version 3; Cytel Software Corporation, Cambridge, MA, USA) statistical package was used when appropriate. All other statistical analyses were performed using the Stata statistical package (version 6; Stata Corporation, College Station, TX, USA). In order to correct for imbalances in age and sex between groups, logistic regression was used. The Armitage trend test was used to examine the relationship between genotypes and ordered categories (eg degree of BHR). In order to correct these trend test analyses for potential confounding factors such as age and sex, ordered logistic regression models were applied.

## Results

Determination of *GSTP1* genotypes

Using the two PCR assays it was possible to identify all genotypes unambiguously, except 24 heterozygotes for the Ile105→Val105 and Ala114→Val114 substitutions, which could have been *GSTP1* AC or BD. Accordingly, ARMS PCR was used to identify these genotypes. Banding patterns are shown in Supplementary Figure 1.

The frequencies of *GSTP1* genotypes are shown in Table 2. In 191 persons, none had BD, CD or DD, reflecting the rarity of *GSTP1*\*D. The frequencies of *GSTP1* genotypes achieved Hardy-Weinberg equilibrium. As reported previously [14], we found significant ( $P < 0.0001$ ,  $\chi^2_4 = 50.9$ ) linkage disequilibrium between Ile105 and Ala114 and between Val105 and Val114.

Association of *GSTP1* genotype with atopic indices

Table 2 shows the association of *GSTP1* genotypes with skin test positivity or IgE level. The frequencies of *GSTP1* BB and BC were reduced in individuals with at least one positive skin test (2.5 and 1.7%, respectively) as compared with individuals who were skin test negative (5.6 and 9.9%, respectively). Although the frequency of *GSTP1* BB was not significantly lower in the persons with positive skin tests (odds ratio [OR] 0.44;  $P = 0.297$ ) or with IgE levels of 100 IU/ml or less, *GSTP1* BC was significantly associated with skin test negativity (OR 0.17;  $P = 0.031$ ; Table 2). There were no significant associations between *GSTP1* genotypes and IgE level, although all individuals with *GSTP1*\*B had IgE levels of 100 IU/ml or less.

### Association of *GSTP1* genotypes with airway obstruction/reactivity

Table 2 shows the frequencies of *GSTP1* genotypes in relation to degree of airflow obstruction and BHR. The *GSTP1* AA frequency increased with severity of airway reactivity/obstruction, whereas BB and BC frequencies displayed a reverse trend. Compared with *GSTP1* AA, trend test analysis across the four groups revealed a significant decrease in frequency of *GSTP1* BC (and *GSTP1* BB, albeit not significant) with increasing airway reactivity/obstruction ( $P=0.031$ ), indicating a protective effect. This effect remained significant after correction for age and sex ( $P=0.022$ ) using ordered logistic regression analysis.

### Association of *GSTP1* genotype with presence of asthma

We further examined the association of *GSTP1* genotypes with the clinical presence of asthma, as defined using the recognized cutoff of  $<8$  mg/ml methacholine [15] on bronchial challenge (groups 1 and 2 versus groups 3 and 4). The frequency of *GSTP1* AA was increased in groups 3 and 4 (44.9%) as compared with groups 1 and 2 (28.8%), whereas *GSTP1* BB and BC frequencies were lower in groups 3 and 4 (2.0 and 2.0%, respectively) than in groups 1 and 2 (5.5 and 8.2%, respectively). Thus, *GSTP1* BC was associated with a sixfold reduction in asthma risk compared with *GSTP1* AA (OR 0.16, 95% confidence interval [CI] 0.03–0.86;  $P=0.032$ ). This association remained significant after correction for age and sex (OR 0.15, 95% CI 0.03–0.80;  $P=0.027$ ).

### Discussion

We showed that the 105 substitution in *GSTP1* is associated with atopy and BHR [5]. We have now extended these observations by examining whether the 114 substitution modulates this effect. We determined the *GSTP1* genotype using two reported PCR assays for each polymorphic site. However, these assays do not differentiate *GSTP1* AC and BD. Accordingly, we developed an ARMS assay to allow identification of all genotypes.

*GSTP1* is of particular interest in asthma, because chromosome 11q13 is associated with its clinical phenotypes: atopy and BHR [16]. Several candidates (high affinity IgE receptor, clara cell secretory protein) have been identified, although the encoding genes do not account for the strength of the linkage to this region [16]. We proposed that *GSTP1* is another candidate gene in this region [17], although it is of course possible that another gene may be the target, and that the observed associations with *GSTP1* genotype reflect linkage disequilibrium with this gene. Thus, the efficiency of detoxification of reactive oxygen species products determined by polymorphism in *GSTP1* may influence the development and/or severity of BHR and asthma.

We found that the frequency of *GSTP1* AA was increased in patients with established asthma. Similar findings have been found in other proinflammatory conditions, such as chronic obstructive pulmonary disease [18] and multiple sclerosis [19]. However, studies in rheumatoid arthritis [20] and basal cell carcinoma [12] show that *GSTP1* Ile105/Ile105 is protective, whereas Val105/Val105 is associated with a worse outcome. Thus, *GSTP1*-associated risk is likely to be disease-dependent, possibly reflecting differences in relevant substrates.

The data reported here also show that the frequencies of *GSTP1* BC and BB were reduced in asthmatic persons, thus indicating a protective effect. This supports the view that there is little additional effect on disease risk from the Ala114→Val114 substitution, although the number of individuals with some genotypes was small and larger numbers would be required to confirm this observation. The advantage with using this ARMS assay is that it is possible to discriminate between genotypes AC and BD. It relies on a relatively large fragment (998 base pairs), however, and is therefore not suitable for genotyping of archival DNA. Thus, this technique is applicable for studies into population genetics.

In conclusion, we described an ARMS assay to identify genotypes resulting from the A, B, C and D alleles of *GSTP1*, and provided data on the effect of *GSTP1* genotype on asthma risk. Our findings suggest that *GSTP1*\*B and *GSTP1*\*C confer similar protective effects. However, large patient groups are required to identify differential effects between *GSTP1*\*B, *GSTP1*\*C and *GSTP1*\*D.

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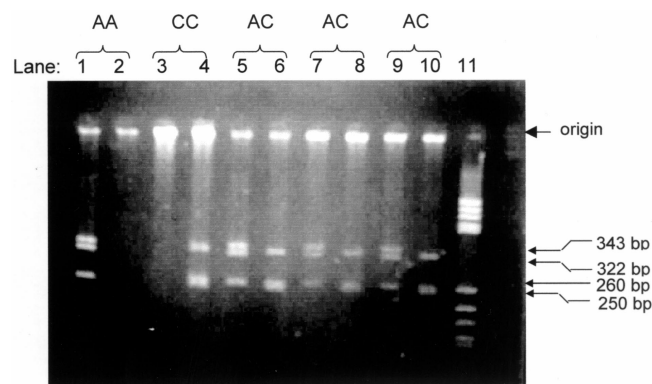
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## Supplementary material

### Supplementary Figure 1



Lanes 1, 3, 5, 7 and 9 include the amplified product from Ala114 specific primer, whereas lanes 2, 4, 6, 8 and 10 include the product from the Val114 specific primer. PCR products from either primer give, after digestion with *Bsm* AI, fragments of either 343, 322, 260 and 73 base pairs for the Ile105 allele, or 322, 260, 250, 93 and 73 base pairs for the Val105 allele. Lane 11, molecular weight markers, pBR Hae III. PCR products were visualized in 2% agarose gels.